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THE CONTRIBUTION OF NITRATE RESPIRATION TO THE ENERGY BUDGET OF THE SYMBIONT-CONTAINING CLAM *LUCINOMA AEQUIZONATA*: A CALORIMETRIC STUDY

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Summary

Heat production and nitrate respiration rates were measured simultaneously in the gill tissue of *Lucinoma aequizonata*. This marine bivalve contains chemoautotrophic, intracellular, bacterial symbionts in its gill tissue. The symbionts show constitutive anaerobic respiration, using nitrate instead of oxygen as a terminal electron acceptor. An immediate increase in heat production was observed after the addition of nitrate to the perfusion medium of the calorimeter and this was accompanied by the appearance of nitrite in the effluent sea water. The nitrate-stimulated heat output was similar under aerobic and anaerobic conditions, which is consistent with the constitutive nature of nitrate respiration. The amount of heat released was dependent on the concentration of nitrate in the perfusion medium. At nitrate concentrations between 0.5 and 5 mmol l^{-1}, the total heat production was increased over twofold relative to unstimulated baseline values. A mean (±S.E.M.) experimental enthalpy of -130 ± 22.6 kJ mol^{-1} nitrite (N=13) was measured for this concentration range.

Key words: calorimetry, symbiosis, nitrate respiration, chemoautotrophy, clam, *Lucinoma aequizonata.*

Introduction

The marine bivalve *Lucinoma aequizonata* is one of a recently discovered group of marine invertebrates which form an association with chemoautotrophic bacteria (Cavanaugh, 1985; Fisher, 1990). These types of animals are found in diverse marine habitats, such as deep-sea hydrothermal vents (Cavanaugh *et al.* 1981; Felbeck *et al.* 1981), the Santa Monica sewage outfall (Felbeck, 1983), mangrove swamps (Schweimanns and Felbeck, 1985), seagrass beds (Fisher and Hand, 1984) and the Santa Barbara basin (Felbeck *et al.* 1981). *Lucinoma aequizonata*, like the other invertebrate hosts described so far, has undergone morphological and physiological modifications to accommodate its symbiotic bacteria: the animal's gills are the symbiont-containing organ; they are greatly enlarged and constitute about 30 % by mass of the tissue of the animal (Distel and Felbeck, 1987). The digestive system is reduced almost to being non-functional (Allen, 1958). The symbiotic bacteria are localized intracellularly in specialized cells of the gills, termed bacteriocytes. The bacteria provide nutrition to the animal by translocation of low-molecular-mass carbon compounds (Distel and Felbeck, 1988) and through intracellular digestion of bacteria by the gill cells (Boetius and Felbeck, 1995). Despite many attempts, the symbionts have not been cultured and their metabolic, physiological and genetic properties are just beginning to be understood. The symbionts fix CO_2 and use a reduced sulfur compound as an electron donor for respiration; the electron acceptor is commonly oxygen or nitrate.

The respiratory reduction of nitrate is a form of anaerobic respiration common in bacteria, but which has never been found in higher organisms (Payne, 1981; Stewart, 1988; Stouthamer *et al.* 1980). Nitrate is used as an alternative electron acceptor and may be reduced to nitrite (nitrate respiration) or nitrogen gas (denitrification). Nitrate respiration is energetically less efficient than oxygen respiration, but it is superior to other forms of anaerobic metabolism, such as fermentation, or to respiratory reduction of other inorganic electron acceptors (e.g. sulfate and bicarbonate) (Thauer *et al.* 1977). The symbionts of *L. aequizonata* are exceptional in that nitrate respiration has completely replaced oxygen respiration. As a result, nitrate respiration is constitutive and, therefore, not inhibited by oxygen (Hentschel *et al.* 1993; Hentschel and Felbeck, 1995). The preferred use of nitrate is thought to provide a selective advantage to the clam in its natural environment, because the animal does not have to compete with its symbionts for oxygen. In its natural habitat, the clam

is exposed to permanently hypoxic conditions; the oxygen level is equivalent to less than 5% air saturation (5–8 μmol l^{-1}), and the mud in which the animals live in shallow burrows becomes anaerobic below the first few millimeters (Cary *et al.* 1989).

The purpose of this paper is to quantify nitrate respiration in this symbiosis. A useful measure for this aim is heat production. The amount of heat released is the sum of the individual contributions of all the reactions occurring in the organism at a given time. Using calorimetry, minute changes in the state of the organism can be traced by measuring changes in heat production (Forrest, 1972; Gustafsson, 1987, 1991). Heat production has been measured in the symbiont-bearing bivalve *Solemya reidi* (Doeller *et al.* 1990). Heat production approached zero in this organism under anaerobic conditions, but remained elevated when thiosulfate, the electron donor for symbiont metabolism in this case, was added to the medium. The authors attributed the excess heat produced to stimulation of symbiont metabolism. If the extra heat production was indeed due to the symbionts, then which substrate served as the respective electron acceptor? So far, the symbionts of *S. reidi* are only known to respire oxygen and nitrate, neither of which was present in the incubation medium.

For our purposes, calorimetry offers several advantages over other biochemical methods: (i) it is highly sensitive, allowing the measurement of minute changes which are below detection limits with more conventional methods; (ii) it is non-invasive, thus allowing the measurement of symbiont heat production while they reside inside the host tissue; (iii) it is non-specific, therefore providing information about the overall response of the tissue to nitrate. In the present study, calorimetry was used to investigate the following questions: does nitrate respiration in the symbionts of *L. aequizonata* result in measurable heat production and, if so, how does this compare with heat production of the animal tissue. These data provide information about the quantitative importance of symbiont nitrate respiration to the overall metabolism of *L. aequizonata*.

Materials and methods

Animal collection and maintenance

Lucinoma aequizonata (Lucinidae) were collected by Otter trawl at a depth of 510±10 m in the Santa Barbara Basin, California, USA, during two cruises in October 1992 and February 1994. Immediately after collection, the animals were placed in chilled sea water (6 °C) for transport to the laboratory. Upon return to the laboratory, the clams were maintained in 80 l aquaria containing artificial sea water (Instant Ocean, Aquarium Systems, Mentor, OH, USA) at 6 °C. All experiments were performed within 2 weeks of collection.

Calorimetry

Direct calorimetry was performed with an LKB 2277 thermal activity monitor (Thermometric AB, Taertaella, Sweden) equipped with a 3.5 ml stainless-steel perfusion chamber. Artificial, nitrate-free sea water (Instant Ocean 35‰, 0.45 μm filtered) was used as a perfusion medium. The flow rate was set at 15 ml h^{-1}. Temperature was maintained at 23±0.0002 °C and regulated by the internal thermostat. All flow lines for gases and sea water were made of Viton tubing and stainless steel. A static calibration was performed with an external heat source of 300 μW. The baseline drift was determined and found to be less than 1 μW during the experiment. Data points were collected every 60 s, generally for 15–20 h, and stored on an IBM XT personal computer and on a strip chart recorder. When the data were time-corrected by the calibration unit of the calorimeter, time constants were 600 s and 18 000 s. After every two runs, the system was cleaned, freshly coated with Sigmacote (Sigma) to prevent corrosion, and subjected to a dynamic calibration.

Even though the clams live at 6 °C in their natural habitat, the experiments could only be performed at 23 °C as a result of experimental constraints. We have tested the tissue stability by maintaining gill tissues at both 6 and 23 °C. The elevated temperatures did not obviously harm the tissue within the timeframe of our experiments: for example, nitrate respiration rates were linear over the experimental period at both temperatures, but were 2.5-fold higher at the elevated temperature (N=5, data not shown).

Experimental procedure

The color and consistency of the gills is an indicator of the well-being of the animal. For this series of experiments, only yellow gills (indicating the presence of elemental sulfur deposits, considered ‘healthy’) were used (Vetter, 1985). Gill demibranchs were excised, rinsed and the wet mass was determined. One demibranch (0.2 –0.35 g wet mass) was used for each experiment. After a stable aerobic heat output had been established, data collection was initiated. Nitrate, at concentrations of 30 μmol l^{-1} to 5 mmol l^{-1}, was then added to the perfusion medium. At the end of the experiment, the gill tissue was dried (at 60 °C, for 48 h) and the dry mass was determined.

Chemical analyses

Oxygen concentrations were measured continuously using a Cyclobios TwinFlow Respirometer (Gnaiger, 1983) which was coupled *via* stainless-steel tubing to the calorimeter. Oxygen concentrations in the perfusion medium were measured before and after passage through the animal chamber by polarographic oxygen sensors and were recorded on a strip chart recorder (Linear 1200). The limits of oxygen detection were below 0.05% air saturation. Anaerobic conditions were established by deoxygenating the seawater reservoir with nitrogen gas (USP standard grade) and aerobic conditions by bubbling with air prior to and during the experiment.

Nitrite concentrations were determined spectrophotometrically in 15 min intervals in the effluent sea water at 543 nm after reaction with sulfanilamide (1% in 1 mol l^{-1} HCl) and 0.01% (w:v) NED [(*N*-(1-napthyl)-ethylenediamine dihydrochloride] (Gieskes and Peretsman, 1986).

Table 1. *Calorimetric/respirometric ratios of* Lucinoma aequizonata *gill tissues at various nitrate concentrations*

					Symbiont heat production	
Nitrate ($mmol\,l^{-1}$)	(N)	Heat production ($\mu J\,min^{-1}\,mg^{-1}$)	Nitrate respiration ($nmol\,min^{-1}\,mg^{-1}$)	C/R ratio ($kJ\,mol^{-1}$ nitrite)	Total ($\mu W\,mg^{-1}$)	Increase over gill tissue heat production (%)
0.03	1	0.8	0.026	31	0.01	2
0.1	3	3.5±0.78	0.038±0.01	92±19	0.06±0.01	8
0.5	1	25.9	0.19	136	0.43	105
1	5	29.3±2.7	0.226±0.02	130±21	0.47±0.05	88
2	2	47.9	0.26	184	0.8	74
		43.3	0.43	101	0.7	92
5	1	45.6	0.45	101	0.76	100

Values are means ± S.E.M.; where N>2.
Total symbiont heat production was calculated after subtracting the gill tissue heat contribution as the baseline.
The symbiont heat production was calculated as the percentage increase over the gill tissue anaerobic metabolic rate.
C/R ratio, calorimetric/respirometric ratio, which is the mean experimental enthalpy for nitrate-stimulated metabolism.

Results

Eight independent calorimetric experiments were performed, the results of which are summarized in Table 1. Typical calorimetric runs using gill tissues are shown in Figs 1 and 2. After a stable aerobic baseline had been established, the conditions were made anaerobic. At certain time points, various concentrations of nitrate (30 $\mu mol\,l^{-1}$ to 5 $mmol\,l^{-1}$) were added. This resulted in an immediate increase in heat production which was accompanied by the appearance of nitrite in the effluent sea water. With increasing nitrate concentrations, a stepwise increase in heat production was observed. The concentration of nitrite in the sea water followed the same pattern. After withdrawal of nitrate from the perfusion medium, the heat production decreased to the basic anaerobic level and, following this, aerobic recovery was initiated. Nitrate-stimulated heat production was similar under aerobic (0.33 and 0.46 $\mu W\,mg^{-1}$ dry mass, N=2) and anaerobic (0.32 and 0.41 $\mu W\,mg^{-1}$ dry mass, N=2) conditions and was independent of the oxygen concentration in the ambient sea water (Fig. 3). The rate of heat production was proportional to the rate of nitrate respiration and was dependent on the concentration of nitrate in the sea water (Fig. 4). Half-maximal stimulation of heat dissipation was achieved at 0.81 $mmol\,l^{-1}$ nitrate. The mean experimental enthalpy for nitrate-stimulated metabolism was −130±22.6 $kJ\,mol^{-1}$ nitrite (N=13, ±S.E.M.) at concentrations between 0.5 and 5 $mmol\,l^{-1}$ nitrate (Table 1).

Gill tissue of *Mytilus edulis* was used as a negative control (Fig. 5). Upon exposure to 1 $mmol\,l^{-1}$ nitrate, there was no change in heat production. Also, in a calorimetric run without gill tissue, addition of 1 $mmol\,l^{-1}$ nitrate or nitrite had no effect on the heat baseline. Other potential electron donors/acceptors (100 $\mu mol\,l^{-1}$ thiosulfate, 1 $mmol\,l^{-1}$ trimethylamine oxide) for symbiont respiration caused no detectable increase in heat dissipation.

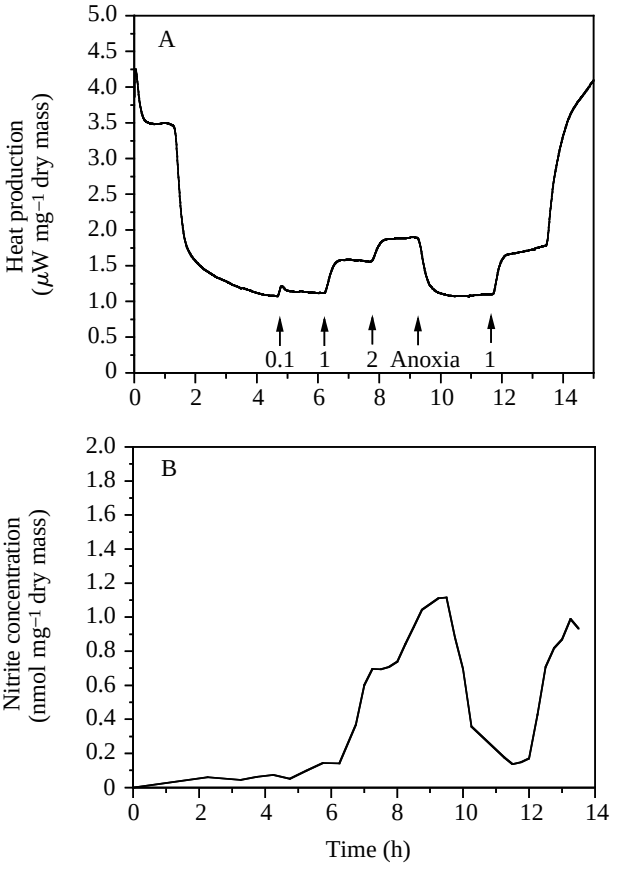

Fig. 1. (A) Heat production of *Lucinoma aequizonata* gill tissue containing intracellular symbionts in the presence of nitrate. After an aerobic baseline had been established, perfusion with N_2-equilibrated, anoxic sea water was started (t=1.5 h). The arrows indicate the stepwise addition of 0.1 $mmol\,l^{-1}$, 1 $mmol\,l^{-1}$ and 2 $mmol\,l^{-1}$ nitrate under anoxia, followed by a 2 h period of anoxia without nitrate. At t=11.75 h, the addition of 1 $mmol\,l^{-1}$ nitrate was repeated. At t=13.75 h, perfusion with oxygenated sea water began. (B) Simultaneous measurements of nitrate respiration measured as the concentration of nitrite in the effluent sea water.

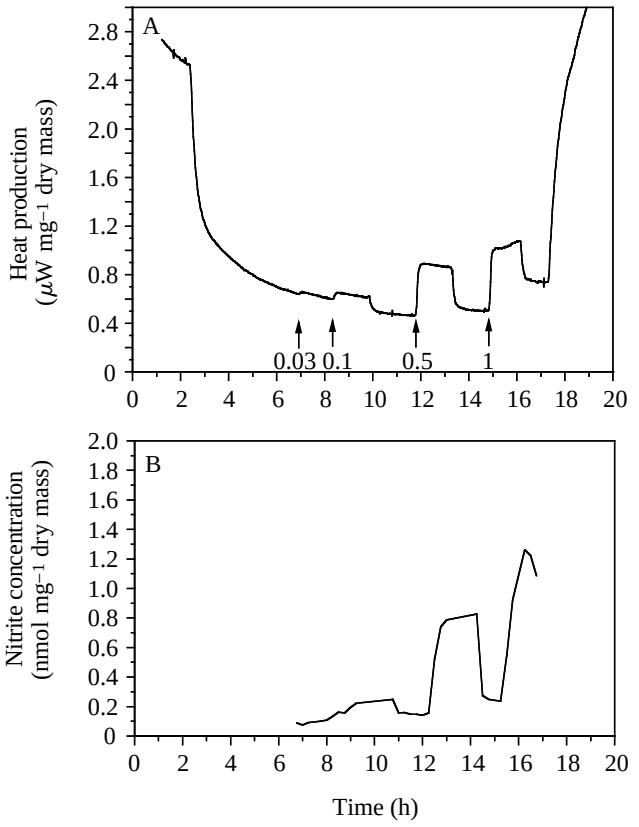

Fig. 2. (A) Heat production of *L. aequizonata* gill tissue in the presence of various concentrations of nitrate. After an aerobic baseline had been established, perfusion with N_2-equilibrated, anoxic sea water was started (t=2.5 h). The arrows indicate the stepwise addition of 0.03, 0.1, 0.5 and 1 mmol l^{-1} nitrate under anoxic conditions for 1.5 h each, followed by a nitrate-free period (except after the 0.03 mmol l^{-1} addition). At t=17.5 h, the aerobic recovery was initiated by perfusion with oxygenated sea water. (B) Simultaneous measurements of the nitrate respiration rate (see Fig. 1).

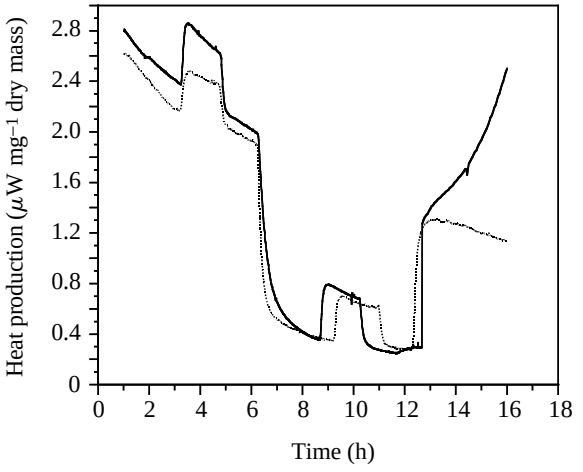

Fig. 3. Heat production of *L. aequizonata* gill tissues from two experiments. Nitrate (1 mmol l^{-1}) was added under aerobic (t=3.5–5 h) and anaerobic (t=9–11 h) conditions. The dotted line indicates the experiment performed in the presence of antibiotics (50 μg ml^{-1} streptomycin, 50 μg ml^{-1} penicillin).

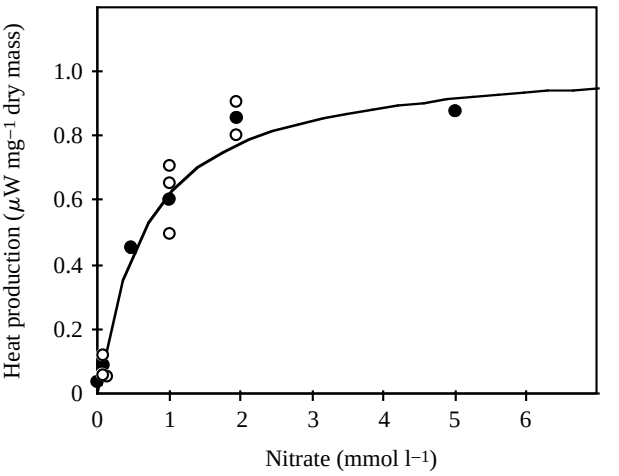

Fig. 4. Heat production of *L. aequizonata* gill tissues under anoxia plotted against the concentration of nitrate in the sea water. The open circles show the original values and the filled circles represent the calculated mean values.

We were concerned about the unrealistically high heat production rate during aerobic recovery at the end of the experiments (Figs 1–3). To ensure that these data were not compromised by free-living contaminants, antibiotics (50 μg ml^{-1} streptomycin, 50 μg ml^{-1} penicillin) were added to one run (Fig. 3). In their presence, a realistic aerobic recovery was measured which resumed the initial aerobic slope. Overall, we decided not to add antibiotics to the experiments because of possible interference with the symbiont metabolism. A significant influence of free-living bacteria on the data can probably be excluded, because (i) the heat production was similar under aerobic and anaerobic conditions, a feature of symbiont respiration but not of the respiration of most free-living bacteria, and (ii) the rate of heat production was linear and not exponential, as would be expected if bacteria were growing in the sea water.

Discussion

The use of nitrate as a substrate for respiration has now been demonstrated in symbionts of various lucinid clams (P. Barnes, personal communication; Hentschel *et al.* 1993), *Solemya reidi* (Wilmot and Vetter, 1992) and also in methanotrophic symbionts of seep mussels (R. W. Lee, personal communication) and the tubeworm *Riftia pachyptila* (Hentschel and Felbeck, 1993). Nitrate respiration appears to be common in these symbioses. While the physiology of this process (oxygen regulation, use of electron donor, stimulation of CO_2 fixation, etc.) has been studied in some detail, until now it was still unknown whether useful amounts of energy could be generated using nitrate as an electron acceptor. We compared the heat production of symbiont nitrate respiration with the heat production of the equivalent animal tissue metabolism. The symbiosis of *L. aequizonata* is particularly suited for this purpose because one can clearly distinguish

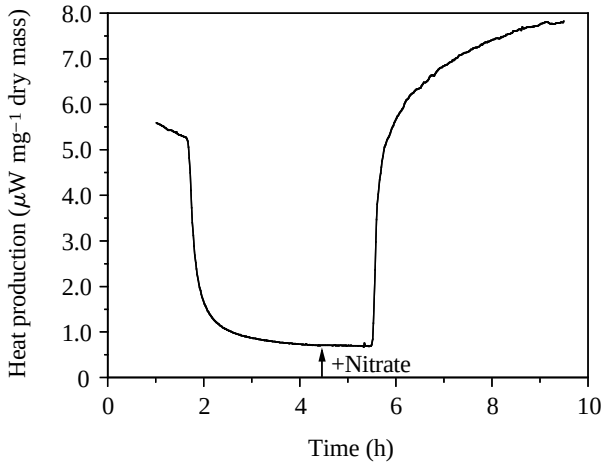

Fig. 5. Heat production of *Mytilus edulis* gill tissue. 1 mmol l^{-1} nitrate was added at t=4.5 h (arrow) during anaerobic conditions. Aerobic recovery was initiated at t=5.5 h.

between animal tissue metabolism and symbiont metabolism. The symbionts respire nitrate only, but not oxygen, and the animal uses oxygen, but never nitrate. This separation of electron acceptors is unique to *L. aequizonata;* all other symbionts investigated in this regard respire oxygen as well as nitrate (Hentschel and Felbeck, 1993, 1995; Wilmot and Vetter, 1992).

When nitrate was added to the perfusion medium, an immediate increase in heat production was observed, which was accompanied by the appearance of nitrite in the effluent sea water. Since only the symbionts respire nitrate, this increase in heat production reflects a stimulation of the symbiont metabolism and all metabolic pathways which depend directly or indirectly on nitrate. These include electron transport in the symbiont respiratory chain, ATP generation and all cellular processes linked to it. Accordingly, in the absence of nitrate, heat production is due entirely to the host's metabolism. It has previously been shown that heat production ceases in cultures of free-living bacteria as soon as the electron acceptor is depleted (Reiling and Zuber, 1983; Samuelsson *et al.* 1988). The rates of nitrate-stimulated heat production are similar under aerobic and anaerobic incubation conditions (Fig. 3). This is consistent with previous results showing that oxygen does not regulate nitrate respiration (Hentschel and Felbeck, 1995).

The intriguing question of how nitrate enters the cells has not yet been addressed. Nitrate is a negatively charged anion (NO_3^-) and must be transported against an electrochemical gradient in order to enter the cytosol. Animal tissues are not known to take up nitrate or to metabolize it in any way. However, the symbioses present a novel situation where bacteria residing inside the tissues require nitrate for their metabolism. Since the reduction of nitrate to nitrite occurs on the cytosolic side of the bacterial membrane, nitrate must cross the host membrane first, then penetrate the bacteriocyte and finally enter the symbionts. Nitrite must be excreted in the same fashion. Even though nitrate reaches the site of its reduction, the mechanisms are not understood.

Our calorimetry experiments show a correlation between the concentration of nitrate in the ambient sea water and the rates of nitrate respiration and heat production. This finding was surprising because it has not been observed in purified symbiont suspensions. At nitrate concentrations around 0.5–2 mmol l^{-1}, the maximum stimulation of heat production was observed, and this leveled off at 2–5 mmol l^{-1} nitrate (Fig. 4). At the environmental nitrate concentration (30 μmol l^{-1}), the rates were much lower. Apparently, the gill has a low affinity for nitrate, because half-maximal rates of nitrate utilisation were achieved at 0.81 mmol l^{-1}. Since the data are based on the rate of nitrite excretion, any of the processes involved, e.g. nitrate uptake, reduction or excretion, could be a rate-limiting factor.

It is possible that the gill simply forms a diffusion barrier and is, therefore, responsible for the low affinity for nitrate. However, results so far point towards an unusual mechanism of nitrate uptake as a more likely explanation in these types of symbioses. For example, the concentrations of nitrate and nitrite in *L. aequizonata* gill homogenates are an order of magnitude higher than those in sea water (U. Hentschel, unpublished results). This observation can only be explained by the presence of a concentrating mechanism in the tissue, but it is not yet known whether it is located in the animal compartment or in the symbionts. Nitrate-concentrating mechanisms are known in plant (Lewis, 1986; Ulrich *et al.* 1990) and bacterial (Thayer and Huffaker, 1982) assimilation, but they have, to our knowledge, never been demonstrated in higher organisms. In addition, a nitrate-concentrating mechanism for dissimilatory purposes was recently demonstrated in the marine chemoautotrophic bacterium *Thioploca* sp., which can concentrate up to 500 mmol l^{-1} nitrate inside vacuoles (Fossing *et al.* 1995). *Thioploca* sp. respire nitrate to nitrogen gas using sulfide as an electron acceptor. More evidence for an unusual mode of nitrate uptake comes from the hydrothermal vent tubeworm *Riftia pachyptila*, which harbors its symbionts in the trophosome inside the coelomic cavity. The nitrate concentrations in the animal blood exceeded those in the ambient sea water by 20- to 50-fold (U. Hentschel, in preparation). Also, Lee and Childress (1994) have shown that $^{15}NO_3^-$ enters the animal tissue, but it is not known whether nitrate is taken up actively or by passive diffusion. Further studies are required to elucidate the possible mechanisms of nitrate uptake and concentration in these symbiotic associations.

A considerable body of information now exists on the analysis of heat production during the metabolism of microorganisms (Beezer, 1980; Gustafsson, 1987, 1991). However, the situation of the symbionts in this study is in many ways different from those in the published literature. Owing to their lifestyle as obligate symbionts, they more closely resemble cell organelles, with the exception being that they are restricted to certain tissues. Their metabolic rates should be compared neither with the high rates of growing bacteria nor

with the low maintenance energies of stationary-phase bacteria (Pirt, 1987).

Thus, in order to assess the quantitative contribution of the symbionts to their respective hosts, one needs to obtain estimates of both symbiont and host metabolic rates. These values have been difficult to quantify, because aposymbiotic hosts do not exist and the symbionts cannot be cultured. When the symbionts are purified, their metabolic rates may not reflect the situation *in vivo*. Nitrate respiration in *L. aequizonata* is well suited for a differentiation of heat flows using calorimetry. In the presence of nitrate, the heat output is the sum of animal and symbiont heat production, while in the absence of nitrate, heat production results only from the animal. Once the animal metabolism is subtracted out as the baseline, then the area integrated under the peak reflects the symbiont heat production during that period (Table 1). Thus, a partitioning of symbiont and animal heat flow can be obtained based on which electron acceptor (oxygen, nitrate) is available. From the data presented in Table 1, the individual contributions of host and symbionts to the overall energy flow can be calculated. The nitrate-stimulated heat production resulted in increases of 2 % (30 μmol l^{-1} nitrate) and 74–105 % (0.5–5 mmol l^{-1} nitrate) under anaerobic conditions above the clam gill heat production. Under aerobic conditions, the symbiont heat production added 10–15 % to the animal's overall heat dissipation.

Alternatively, the symbiont and host contributions may be compared, in metabolic terms, if the generation of ATP is used as the unit of comparison. The rate of catabolic ATP generation is specific for each pathway and has been calculated from experimentally established coupling coefficients (Gnaiger, 1983). The stoichiometric coupling coefficient for fully dissipative metabolism is 6.17 ATP/O_2. Therefore, a gill respiration rate of about 12 nmol O_2 mg^{-1} dry mass h^{-1} is equivalent to an ATP production rate of 74 nmol ATP mg^{-1} dry mass h^{-1}. If the clam metabolism is completely anaerobic, ATP production can be inferred from published data on *Mytilus edulis* (Kluytmans *et al.* 1977). After 24 h of anaerobiosis, the key end-product of anaerobic metabolism, succinate, had accumulated in gill tissues at a rate of 1.14 nmol succinate mg^{-1} gill dry mass h^{-1}. Since the stoichiometric coupling coefficient is 2.75 ATP/succinate, a total of 3.1 nmol ATP mg^{-1} gill dry mass h^{-1} would have been produced, by analogy, in *L. aequizonata* gills under anaerobic conditions.

The respiration of nitrate to nitrite by the symbionts is coupled to the oxidation of elemental sulfur. The stoichiometric coupling coefficient for this pathway has, to our knowledge, not been published. In *Thiobacillus denitrificans*, denitrification with thiosulfate as the electron donor results in 1.18 ATP/nitrate (Timmer-ten Hoor, 1976). Therefore, the symbiont nitrate respiration rate of 1.56–13.2 nmol mg^{-1} dry gill mass h^{-1} at 0.03–1 mmol l^{-1} nitrate would be approximately equivalent to 1.8–15.6 nmol ATP mg^{-1} dry gill mass h^{-1}. These values are likely to be overestimates for the clam gill, because nitrate is only respired to nitrite and not to nitrogen gas, and sulfur rather than thiosulfate is the electron donor.

Using these values as a first approximation, the animal tissue and symbiont metabolic rates can be compared in terms of the generation of ATP. Under anaerobic conditions, the rates of clam (3.1 nmol ATP mg^{-1} gill dry mass h^{-1}) and symbiont (1.8–15.6 nmol ATP mg^{-1} dry mass h^{-1}) ATP production are of the same order of magnitude. Under aerobic conditions, the clam metabolism would produce 74 nmol ATP mg^{-1} gill dry mass h^{-1}, a rate significantly higher than the comparable symbiont ATP production rate. The heat production reflects the same general pattern, i.e. the symbiont-generated heat is about equal to the gill heat production under anaerobic conditions, whereas in the presence of oxygen, the symbiont heat production is only 10–15 % of the gill heat production. Taken together, these values indicate that symbiont nitrate respiration accounts for a significant portion of the overall energy budget of the symbiotic system. It appears to be especially important for energy generation under anaerobic conditions, a situation which the animals are likely to encounter in their natural environment.

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